JOURNAL OF VIROLOGY, July 1996, p. 4825–4828 Vol. 70, No. 7
0022-538X/96/$04.00+0

Two Separate Envelope Regions Influence Induction of Brain Disease by a Polytropic Murine Retrovirus (FMCF98)

KIM J. HASENKRUG,* SHELLY J. ROBERTSON, JOHN PORTIS, FRANK MCATEE, JANE NISHIO, AND BRUCE CHESEBRO

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

Received 19 January 1996/Accepted 9 April 1996

The major determinants involved in neurological disease induction by polytropic murine leukemia virus FMCF98 are encoded by the envelope gene. To map these determinants further, we produced four chimeras which contained neurovirulent FMCF98 envelope sequences combined with envelope sequences from the closely related nonneurovirulent polytropic virus FMCF54. Surprisingly, two chimeric viruses containing completely separate envelope regions from FMCF98 could both induce neurological disease. Clinical signs caused by both neurovirulent chimeras appeared to be indistinguishable from those caused by FMCF98, although the incubation periods were longer. One neurovirulence determinant mapped to the N-terminal portion of gp70, which contains the VRA and VRB receptor-binding regions, while the other determinant mapped downstream of both of the variable regions. Western blot (immunoblot) analyses and immunohistochemical staining of tissue sections indicated that the variations in neurovirulence of these viruses could not be explained by differences in either the quantitative level or the location of virus expression in the brain.

Several murine leukemia viruses (MuLVs) cause neurological diseases in mice, and previous studies have indicated that the *env* genes are critical determinants of disease induction (8, 15, 16, 19, 20, 24). Most of the neurovirulent retroviruses have envelope proteins which utilize the ecotropic receptor for infection of cells. The clinical signs associated with infection by ecotropic MuLVs typically include hind-limb paralysis. These viruses are widely distributed throughout the brain and induce severe neurodegenerative pathology consisting of spongiosis and gliosis (1, 3, 10, 28). We have previously reported on a neurovirulent murine retrovirus, FMCF98, which uses the polytropic rather than the ecotropic receptor (4, 20). Infection of newborn mice with FMCF98 produces clinical signs consisting of tremors, ataxia, and death within 1 to 3 months. Virus expression in the brain is restricted primarily to white matter tracts of the cerebellum and cerebellar peduncles, internal capsule, and corpus callosum (20). Despite the severity of disease, FMCF98 produces minimal pathological abnormalities other than gliosis in areas of high virus expression (20).

In the present study, the viral envelope regions involved in the neurovirulence of FMCF98 were mapped by constructing chimeric envelope genes composed of FMCF98 sequences combined with sequences from a closely related polytropic nonneurovirulent clone, FMCF54 (13, 18). To restrict comparisons to the *env* gene sequences, all *env* genes were expressed in the Friend virus background (clone FB29) as previously described (20) to produce Fr98, Fr54, and four viruses with chimeric *env* genes (Fig. 1). Plasmids containing the recombinant retroviruses were transfected into *Mus dunni* cells to produce virus stocks. Each virus spread to confluency as determined by staining of the cultures with envelope-specific monoclonal antibodies (data not shown), and all but Fr98/SA produced virus titers of greater than 10^6 focus-forming units/ml of cell culture supernatant (Table 1). Repeated failures to obtain comparable titers from two separate Fr98/SA clones indicated that its envelope was limiting virus replication in some manner.

Titered stocks of the recombinant viruses were used to infect newborn mice, and at 19 days postinfection, blood samples were assayed to determine levels of plasma viremia as an indication of in vivo replication. Mean titers between 10^4 and 10^5/ml were obtained for all viruses except Fr98/SA, the titer of which was 100-fold lower at this time point (Table 1). Infected animals were monitored for signs of clinical disease, and as described previously (20), the first signs manifested by mice infected with Fr98 were hyperactivity and an accentuated arousal response to noise. The disease progressed within several days to abnormal hind-limb adduction when the mice were lifted by the tail and imbalance characterized by falling over when the mice attempted to crawl up the sides of the enclosure. By 4 weeks postinfection, 100% of the animals infected with Fr98 were neurologically affected (Fig. 2).

Surprisingly, two of the chimeras (Fr98/SE and Fr98/EC) containing completely reciprocal *env* regions from Fr98 (Fig. 1) also induced neurological disease. The clinical signs of mice infected by both chimeras were indistinguishable from those induced by Fr98, although the incubation periods were longer and the incidence of disease was slightly less than 100% (Fig. 2). The induction of disease by chimera Fr98/SE indicated that the 12-amino-acid differences between the Fr54 and Fr98/SE envelope proteins (Fig. 1) were sufficient for conversion to neurovirulence. However, as indicated by Fr98/EC, there was a separate region downstream of the *Eco*RI site which was also sufficient for conversion to neurovirulence. Fr54 and Fr98/EC differ by 40 residues in this region. The failure of Fr98/AC to induce disease suggested that the downstream neurovirulence determinant in Fr98/EC mapped to the 17 amino acid differences between the *Eco*RI and *Avr*II sites (Fig. 1). However, data from the present constructs did not exclude the possibility that the *Avr*II-to-*Cla*I region also contributed to the neurovirulence of Fr98/EC. Even though the fourth chimera, Fr98/SA,

* Corresponding author. Mailing address: Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 363-9310. Fax: (406) 363-9204. Electronic mail address: Kim Hasenkrug@rml.niaid.pc.niaid.nih.gov.

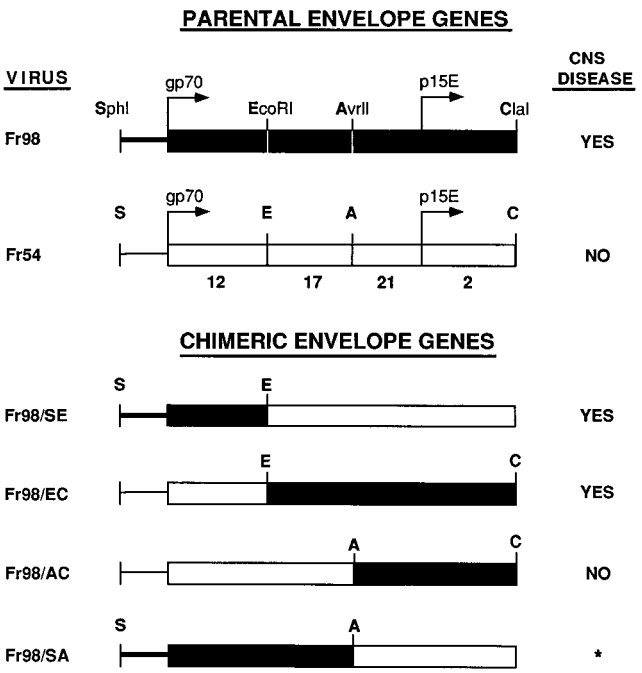

FIG. 1. Schematic diagram of the 2.45-kb *Sph*I-*Cla*I regions of parental and chimeric viruses. Chimeras in the Friend FB29 background are prefixed by Fr and named according to the restriction fragment borders derived from Fr98. The number of amino acid differences between the parental proteins is shown below Fr54 for each *env* region. *, the Fr98/SA chimera produced no neurological symptoms but did not produce high-titered stocks in vitro and replicated at only low levels in vivo (see Table 1). CNS, central nervous system.

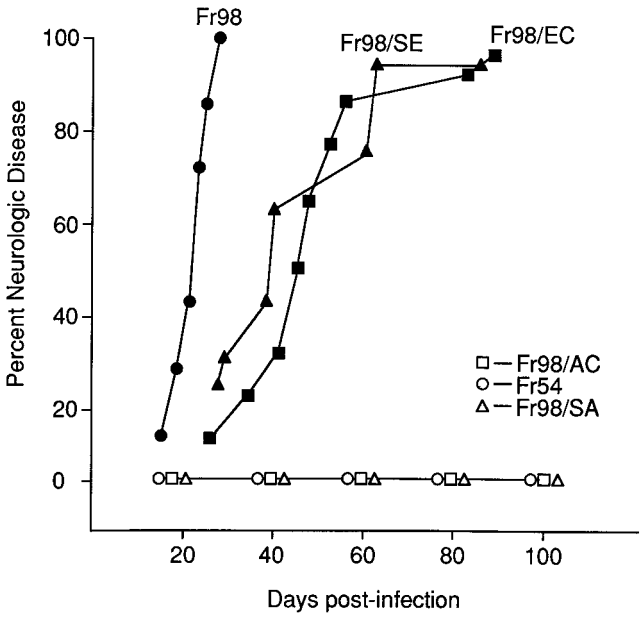

FIG. 2. Development of neurological disease in mice infected with Friend virus expressing various envelope genes. Inbred Rocky Mountain White (IRW) mice (less than 48 h old) were inoculated intraperitoneally with 30 μl of a virus stock (titers are shown in Table 1). The numbers of mice per group were as follows: Fr54B (○), 21; Fr98/SE (▲), 16; Fr98/EC (■), 22; Fr98/SA (△), 26; Fr98/AC (□), 24; Fr98 (●), 7.

contained both of the mapped neurovirulence regions, its failure to induce disease was not unexpected because of its low titers in vivo and in vitro. It has been observed with other neurovirulent viruses that high virus titers in the blood or spleen may be necessary for central nervous system infection (6, 22, 27, 31).

Differences in neurovirulence could be due to variability in the capacity to infect the brain. To determine whether neurovirulence correlated with relative levels of virus production in the brain, we examined viral $p30^{gag}$ protein expression in the cerebellum. We concentrated on the cerebellum because it is the region of the brain most heavily infected by Fr98 (20), and pathology in the cerebellum is consistent with ataxia, the primary clinical sign of Fr98-induced disease. Western blot (immunoblot) analyses were performed on 10% cerebellum homogenates at four time points as previously described (7). The blots were probed for FB29 p30, as this protein was conserved among all of the viruses and could be detected equivalently with a single antiserum (Fig. 3). These analyses revealed higher levels of p30 expression in mice infected with the parental neurovirulent virus, Fr98, than in the other mice. On the other hand, Fr98/AC, which did not cause neurological disease, had little or no detectable p30. However, there was no strict correlation between p30 levels and neurovirulence since the non-

TABLE 1. Virus stock titers and plasma viremia levels

Virus	Virus stock titer[a]	Mean virus titer in plasma (SEM)[b]
Fr54	3.6×10^6	4.8×10^4 (0.7×10^4)
Fr98	1.8×10^6	2.3×10^4 (0.9×10^4)
Fr98/SE	4.2×10^6	1.1×10^5 (0.1×10^5)
Fr98/EC	8.3×10^6	1.5×10^5 (0.1×10^5)
Fr98/AC	2.8×10^6	1.3×10^5 (0.1×10^5)
Fr98/SA	5.4×10^4	1.2×10^3 (0.1×10^3)

[a] Virus stocks were 24-h supernatants from confluently infected *M. dunni* cells obtained as previously described (19), and titers were measured in focus-forming units per milliliter.

[b] Newborn mice were inoculated intraperitoneally with 30 μl of each virus stock. Virus titer in plasma were determined at 19 days postinoculation. The number of mice in each group is shown in the legend to Fig. 2.

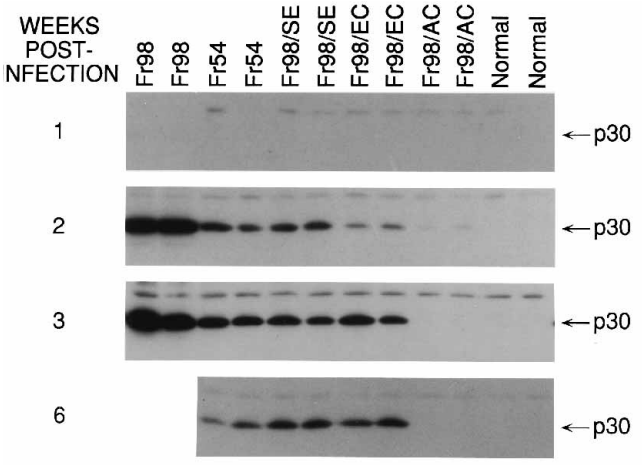

FIG. 3. Western blot analyses of p30 capsid protein. Total cerebellar protein extracts (10% [wt/vol] suspensions) from two mice at each time point were analyzed by Western blotting as previously described (7). Each lane was loaded with the same amount of a suspension. The same blots were also probed for viral envelope protein and gave similar results (data not shown). Two mice from each infection were tested for each time point, except for Fr98 at 6 weeks, since those mice had died or were euthanized.

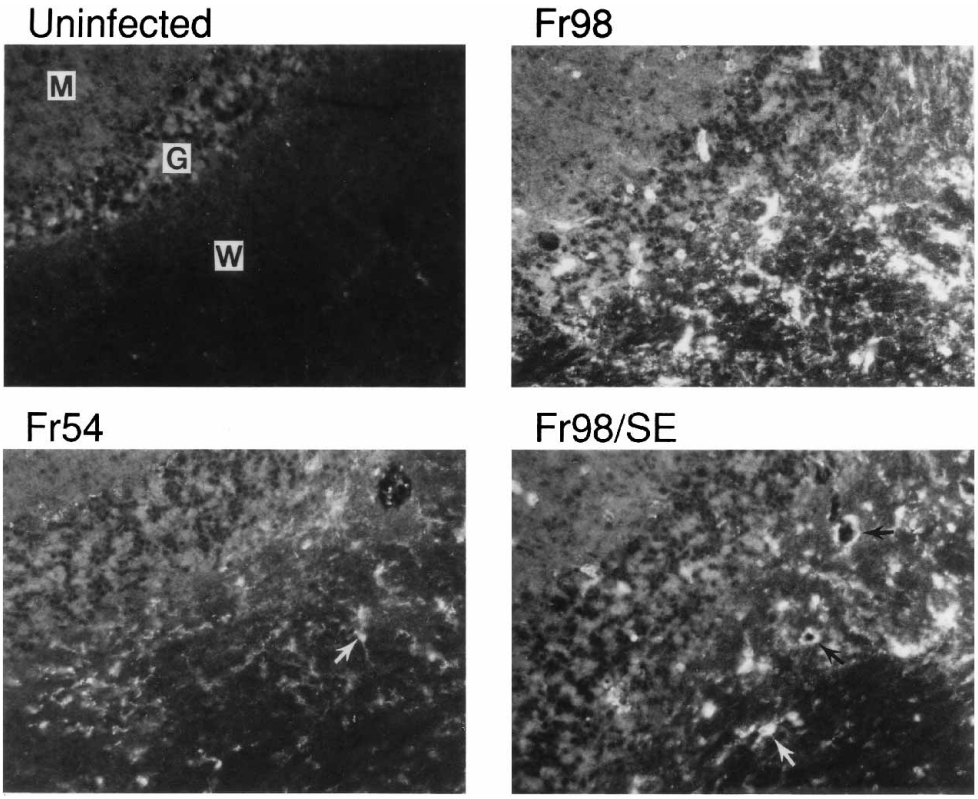

FIG. 4. General distribution of viral antigen in cerebella of virus-infected mice. Fresh frozen sections (4 to 6 μM) of cerebellum were stained for viral envelope glycoprotein by using a polyclonal goat anti-MuLV gp70 serum (kindly provided by Roland Friedrich, Institute of Medical Virology Giessen, Giessen, Germany), followed by a fluorescein isothiocyanate-conjugated donkey anti-goat immunoglobulin G serum (ICN, Biomedicals, Inc.). A low-power coronal view of the cerebellum revealed the three layers of the cerebellar cortex indicated as follows: M, molecular layer; G, granule layer; W, white matter tract. Shown are representative photos of an uninfected mouse, an Fr98-infected mouse, an Fr54-infected mouse, and an Fr98/SE-infected mouse. Black arrows indicate staining in structures with the morphology of capillary endothelial cells. White arrows indicate staining in the parenchyma of the cerebellum. The relative intensity of staining varied from section to section for any given virus and should not be considered quantitative. Sections were viewed on a Nikon Microphot SA microscope. Magnification, ×50.

neurovirulent virus, Fr54, expressed levels of p30 approximately equivalent to those of the neurovirulent envelope chimeras, Fr98/SE and Fr98/EC. Thus, factors in addition to virus expression in the cerebellum appeared to be necessary for disease induction.

The relatively high level of virus expression in the Fr54-infected mouse cerebellum was intriguing since no clinical signs were associated with infection by this virus. A possible explanation for these results is that Fr54 infected capillary endothelial cells of the brain to high levels but did not spread into the brain parenchyma. To investigate this possibility, frozen sections of cerebellum were analyzed by immunofluorescence by using a viral envelope-specific polyclonal antiserum. Results from three of the viruses are shown in Fig. 4. The primary site of infection for all viruses was in white matter tracts. The black arrows point to structures with the morphology of capillaries which are brightly stained for viral antigen. These structures are readily distinguishable from the bulk of staining, which, for all viruses, including Fr54, appeared to be associated with a loose network of glial cell processes and occasional cell bodies (Fig. 4, white arrows) in the white matter. Thus, the failure of Fr54 to induce disease could not be attributed to lack of spread into the parenchyma of the brain. Studies are under way to determine if the specific types of infected glial cells differ between the viruses and whether regions other than the cerebellum might reveal correlates with disease.

The finding that two completely separate envelope regions of Fr98 could each induce neurovirulence was rather unexpected. It is unclear whether the Fr98 neurovirulence determinants acted independently or if interactions with other envelope regions were critical for disease induction. However, the decreased incubation period when both regions were present in clone Fr98 (Fig. 2) suggests that complementary interactions between regions could be important for neurovirulence. The context into which the envelope sequences are introduced is of critical importance. This is illustrated by the phenotype of envelope chimera Fr98/AC, which had levels of expression in the brain completely different from those of either parental virus.

Two separate neurovirulence determinants have also been described in the envelope gene of Moloney MuLV TB mutant *ts*1: one in the amino-terminal half of gp70 which affected the stability and processing of the precursor protein and the other in carboxy-terminal p15E which enhanced replication in the central nervous system (25, 28–30). However, in *ts*1, the determinants could not act independently of each other to induce the same clinical signs (24, 26, 28, 30).

A possible mechanism which could explain why multiple regions of the envelope are responsible for neurovirulence is that one or both of the regions influence recombinations with endogenous retroviral sequences in the mouse genome to create neurovirulent viruses as a secondary effect. Immunofluorescent staining of frozen brain sections from infected mice

was done with monoclonal antibodies 720 and 83A, which specifically react with epitopes encoded in the *Avr*II-to-*Cla*I regions of Fr98 and Fr54, respectively (9). Antibody staining was always consistent with the input viruses, but recombinants which retained the 720 or 83A epitopes or recombinants which comprised only a small fraction of the virus pool would not have been detected.

The similarity of the clinical signs of mice infected with all of these neurovirulent chimeras suggested that the mechanisms of disease induction might be related. Clues regarding those mechanisms might be gleaned from the known functions of the envelope and subregions of the envelope. All of the viruses in this study utilized the polytropic receptor for entry into cells, but subtle variations of envelope sequences could alter the infectibility of different cell types which share the same viral receptor, as has been seen for human immunodeficiency virus (5). The major tropism-determining regions described for MuLV envelopes, VRA and VRB (2), are both located upstream of the *Eco*RI site (Fig. 1). Thus, the neurovirulence of Fr98/SE might require the presence of unique Fr98 sequences at the VRA and VRB variable regions.

Although Fr98 and human immunodeficiency virus induce very different clinical syndromes, in certain respects, Fr98-induced neurological disease may be a useful model for human immunodeficiency virus-associated dementia. As shown here for Fr98, sequence data on both human (21) and simian (14, 24) immunodeficiency viruses suggest that specific envelope sequence variations may be associated with clinical brain infection. Furthermore, in AIDS patients, there is a similar lack of correlation between clinical signs of dementia and viral expression levels, viral localization, and extent of tissue destruction (11, 12, 23). It remains unclear how retroviral envelope determinants affect neuropathogenesis, but it appears that host responses and the genetic background may also play an important role. For example, AKR mice are resistant to neurological disease induction by the MuLV CasBr even though the virus replicates to high levels in the brain (17). The unique ability to control such host responses and genetics in the murine systems may be critical for determining the mechanisms underlying retrovirus-induced central nervous system diseases.

REFERENCES

1. **Andrews, J. M., and M. B. Gardner.** 1974. Lower motor neuron degeneration associated with type C RNA virus infection in mice: neuropathological features. J. Neuropathol. Exp. Neurol. **33:**285–307.
2. **Battini, J. L., J. M. Heard, and O. Danos.** 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses. J. Virol. **66:**1468–1475.
3. **Brooks, B. R., J. R. Swarz, and R. T. Johnson.** 1980. Spongiform polioencephalomyelopathy caused by a murine retrovirus. I. Pathogenesis of infection in newborn mice. Lab. Invest. **43:**480–486.
4. **Buller, R. S., K. Wehrly, J. L. Portis, and B. Chesebro.** 1990. Host genes conferring resistance to a central nervous system disease induced by a polytropic recombinant Friend murine retrovirus. J. Virol. **64:**493–498.
5. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J. Virol. **66:**6547–6554.
6. **Czub, M., F. J. McAtee, and J. L. Portis.** 1992. Murine retrovirus-induced spongiform encephalomyelopathy: host and viral factors which determine the length of the incubation period. J. Virol. **66:**3298–3305.
7. **Czub, S., W. P. Lynch, M. Czub, and J. L. Portis.** 1994. Kinetic analysis of spongiform neurodegenerative disease induced by a highly virulent murine retrovirus. Lab. Invest. **70:**711–723.
8. **DesGroseillers, L., M. Barrette, and P. Jolicoeur.** 1984. Physical mapping of the paralysis-inducing determinant of a wild mouse ecotropic neurotropic virus. J. Virol. **52:**356–363.
9. **Evans, L. H., R. P. Morrison, F. G. Malik, J. Portis, and W. J. Britt.** 1990. A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. J. Virol. **64:**6176–6183.
10. **Gardner, M. B., B. E. Henderson, J. E. Officer, R. W. Rongey, J. C. Parker, C. Oliver, J. D. Estes, and R. J. Huebner.** 1973. A spontaneous lower motor neuron disease apparently caused by indigenous type-C RNA virus in wild mice. J. Natl. Cancer Inst. **51:**1243–1254.
11. **Johnson, R. T., J. D. Glass, J. C. McArthur, and B. Chesebro.** 1996. Quantitation of human immunodeficiency virus in brains of demented and non-demented patients with AIDS. Ann. Neurol. **39:**392–395.
12. **Johnson, R. T., J. C. McArthur, and O. Narayan.** 1988. The neurobiology of human immunodeficiency virus infections. FASEB J. **2:**2970–2981.
13. **Koch, W., W. Zimmermann, A. Oliff, and R. Friedrich.** 1984. Molecular analysis of the envelope gene and long terminal repeat of Friend mink cell focus-inducing virus: implications for the functions of these sequences. J. Virol. **49:**828–840.
14. **Lane, T. E., M. J. Buchmeier, D. D. Watry, D. B. Jakubowski, and H. S. Fox.** 1995. Serial passage of microglial SIV results in selection of homogeneous *env* quasispecies in the brain. Virology **212:**458–465.
15. **Masuda, M., P. M. Hoffman, and S. K. Ruscetti.** 1993. Viral determinants that control the neuropathogenicity of PVC-211 murine leukemia virus in vivo determine brain capillary endothelial cell tropism of the virus in vitro. J. Virol. **67:**4580–4587.
16. **Masuda, M., M. P. Remington, P. M. Hoffman, and S. K. Ruscetti.** 1992. Molecular characterization of a neuropathogenic and nonerythroleukemogenic variant of Friend murine leukemia virus PVC-211. J. Virol. **66:**2798–2806.
17. **McAtee, F. J., and J. L. Portis.** 1985. Monoclonal antibodies specific for wild mouse neurotropic retrovirus: detection of comparable levels of virus replication in mouse strains susceptible and resistant to paralytic disease. J. Virol. **56:**1018–1022.
18. **Oliff, A., L. Collins, and C. Mirenda.** 1983. Molecular cloning of Friend mink cell focus-inducing virus: identification of mink cell focus-inducing virus-like messages in normal and transformed cells. J. Virol. **48:**542–546.
19. **Portis, J. L., S. Czub, C. F. Garon, and F. J. McAtee.** 1990. Neurodegenerative disease induced by the wild mouse ecotropic retrovirus is markedly accelerated by long terminal repeat and *gag-pol* sequences from nondefective Friend murine leukemia virus. J. Virol. **64:**1648–1656.
20. **Portis, J. L., S. Czub, S. Robertson, F. McAtee, and B. Chesebro.** 1995. Characterization of a neurologic disease induced by a polytropic murine retrovirus: evidence for differential targeting of ecotropic and polytropic viruses in the brain. J. Virol. **69:**8070–8075.
21. **Power, C., J. C. McArthur, R. T. Johnson, D. E. Griffin, J. D. Glass, S. Perryman, and B. Chesebro.** 1994. Demented and nondemented patients with AIDS differ in brain-derived human immunodeficiency virus type 1 envelope sequences. J. Virol. **68:**4643–4649.
22. **Prasad, G., G. Stoica, and P. K. Y. Wong.** 1989. The role of the thymus in the pathogenesis of hind-limb paralysis induced by ts1, a mutant of Moloney murine leukemia virus-TB. Virology **169:**332–340.
23. **Sharer, L. R.** 1992. Pathology of HIV-1 infection of the central nervous system. A review. J. Neuropathol. Exp. Neurol. **51:**3–11.
24. **Stephens, E. B., Z. Q. Liu, G. W. Zhu, I. Adany, S. V. Joag, L. Foresman, E. J. Berman, and O. Narayan.** 1995. Lymphocyte-tropic simian immunodeficiency virus causes persistent infection in the brains of rhesus monkeys. Virology **213:**600–614.
25. **Szurek, P. F., P. H. Yuen, J. K. Ball, and P. K. Y. Wong.** 1990. A Val-25-to-Ile substitution in the envelope precursor polyprotein, gPr80^{env}, is responsible for the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1, a mutant of Moloney murine leukemia virus TB. J. Virol. **64:**467–475.
26. **Szurek, P. F., P. H. Yuen, R. Jerzy, and P. K. Y. Wong.** 1988. Identification of point mutations in the envelope gene of Moloney murine leukemia virus TB temperature-sensitive paralytogenic mutant *ts*1: molecular determinants for neurovirulence. J. Virol. **62:**357–360.
27. **Wong, P. K. Y., C. Knupp, P. H. Yuen, M. M. Soong, J. F. Zachary, and W. A. F. Tompkins.** 1985. *ts*1, a paralytogenic mutant of Moloney murine leukemia virus TB, has an enhanced ability to replicate in the central nervous system and primary nerve cell culture. J. Virol. **55:**760–767.
28. **Wong, P. K. Y., and P. H. Yuen.** 1992. Molecular basis of neurologic disorders induced by a mutant, *ts*1, of Moloney murine leukemia virus, p. 161–197. *In* R. P. Roos (ed.), Molecular neurovirology. Humana Press Inc., Totowa, N.J.
29. **Yuen, P. H., D. Malehorn, C. Knupp, and P. K. Y. Wong.** 1985. A 1.6-kilobase-pair fragment in the genome of the *ts*1 mutant of Moloney murine leukemia virus TB that is associated with temperature sensitivity, nonprocessing of Pr80^{env}, and paralytogenesis. J. Virol. **54:**364–373.
30. **Yuen, P. H., E. Tzeng, C. Knupp, and P. K. Y. Wong.** 1986. The neurovirulent determinants of *ts*1, a paralytogenic mutant of Moloney murine leukemia virus TB, are localized in at least two functionally distinct regions of the genome. J. Virol. **59:**59–65.
31. **Zachary, J. F., C. J. Knupp, and P. K. Y. Wong.** 1986. Noninflammatory spongiform polioencephalomyelopathy caused by a neurotropic temperature-sensitive mutant of Moloney murine leukemia virus TB. Am. J. Pathol. **124:**457–468.